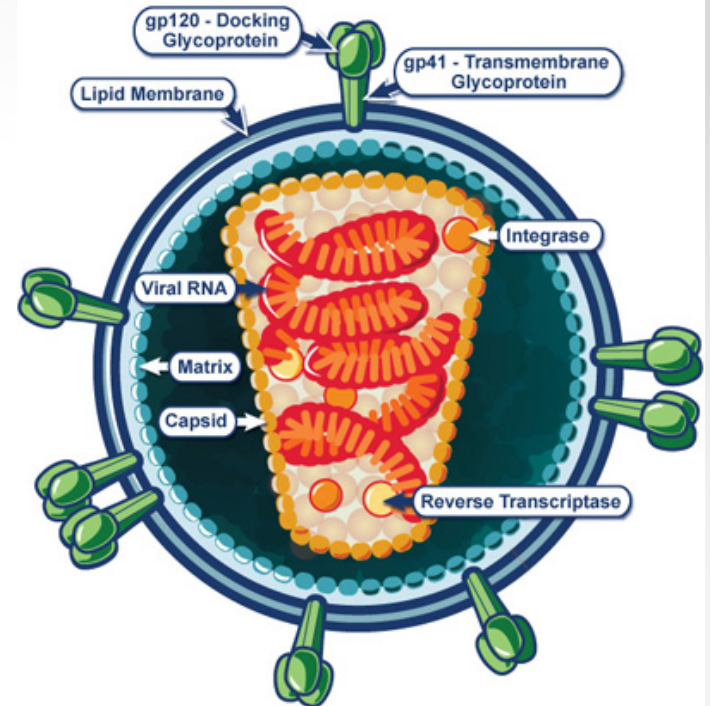


Identification of Mutation(s) in the HIV-1 gp41 Subunit Associated with Neutralization Resistance

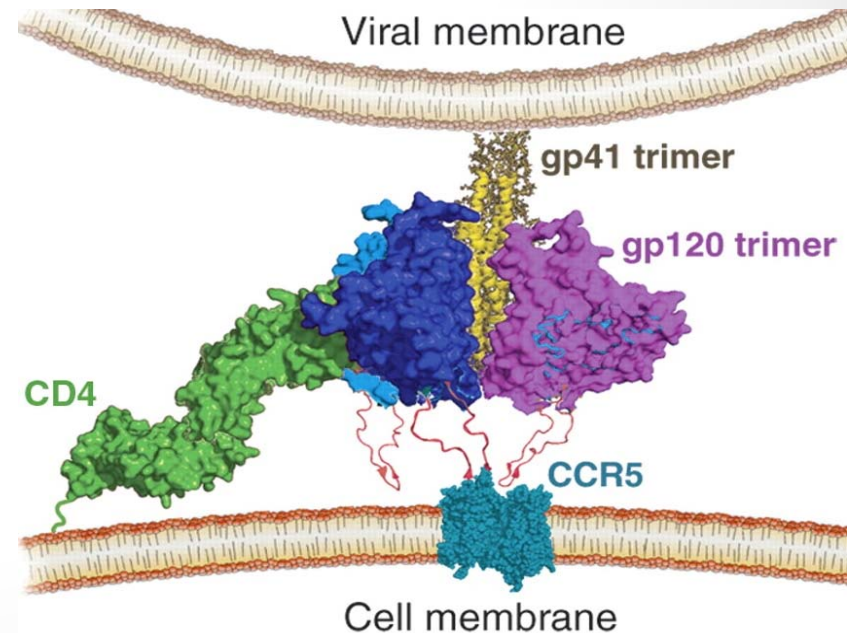
Miah Blomquist

What is HIV-1?

- HIV-1 is an epidemic that affects over 34 million people worldwide.
- HIV-1 causes disease by attacking CD4 T-cells, which are essential to a healthy immune system.
- Viral glycoproteins on the surface on the viral envelope are the proteins the virus uses to enter and infect these T-cells.
- These viral glycoproteins consists of two non-covalently bonded subunits: gp120 and gp41, which associate as a heterotrimer.
- gp120 binds to the CD4 cell receptor inducing a conformation change exposing the coreceptor binding site.



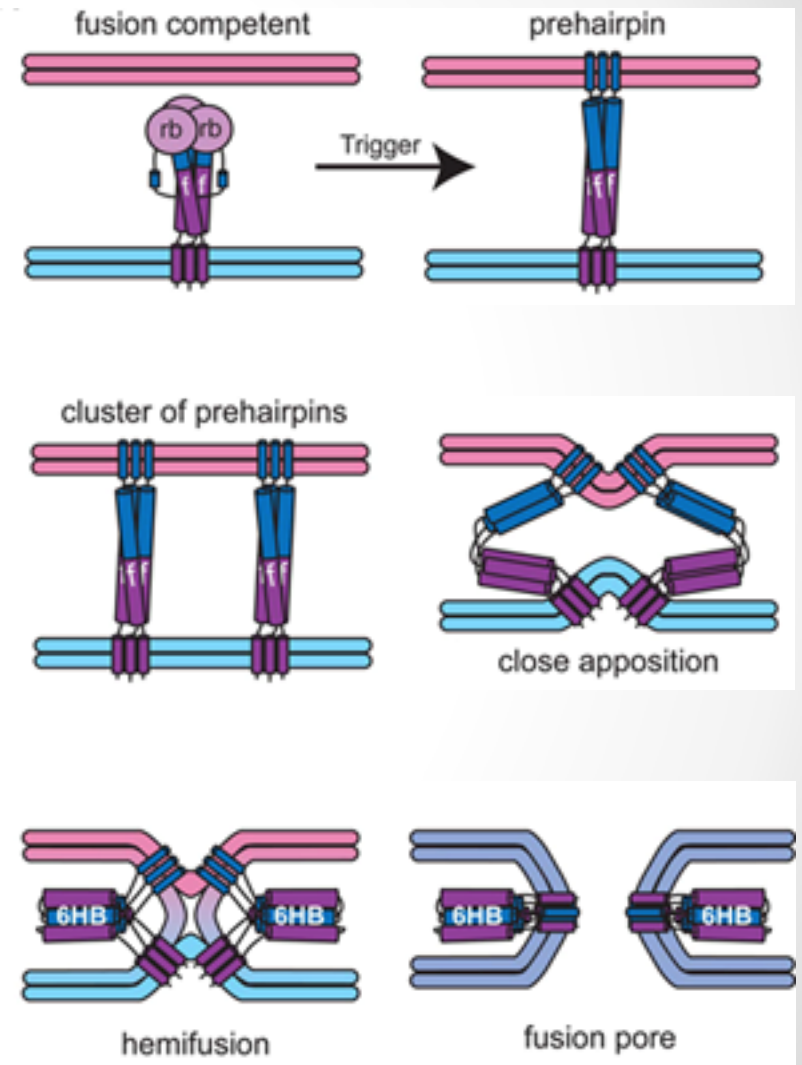
<http://www.niaid.nih.gov/topics/hiv aids/understanding/biology/Pages/structure.aspx>



<http://www.sciencemag.org/content/320/5877/760.figures-only>

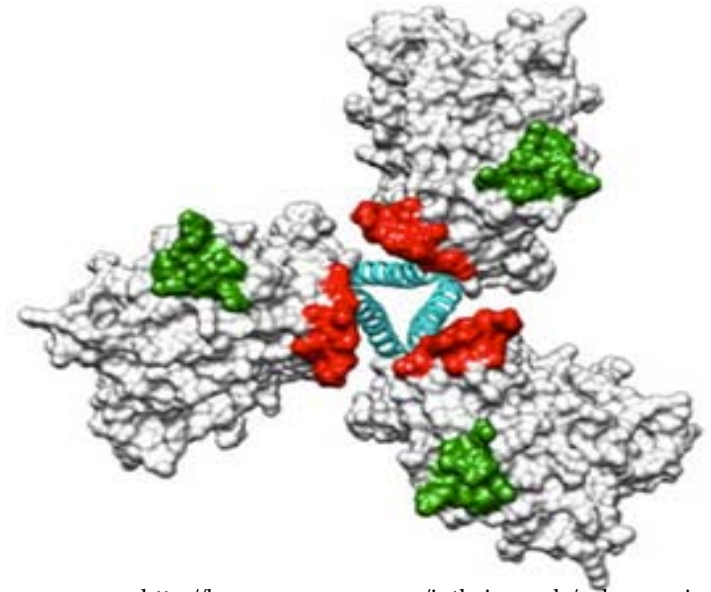
HIV-1 fusion and entry

- Once the gp120 subunit binds to the coreceptor, the gp41 subunit is exposed and undergoes a conformational change, unfolding and inserting into the host cell membrane.
- Once the gp41 subunit trimer is inserted, a cluster of two trimer prehairpins form.
- gp41 trimers then fold onto themselves creating a hairpin-like six helix bundle.
- Trimeric hairpins bring the membranes together, causing the outer membranes to fuse in hemifusion and the inner membrane to fuse, completely joining the two.
- The capsid is then free to enter the host cell

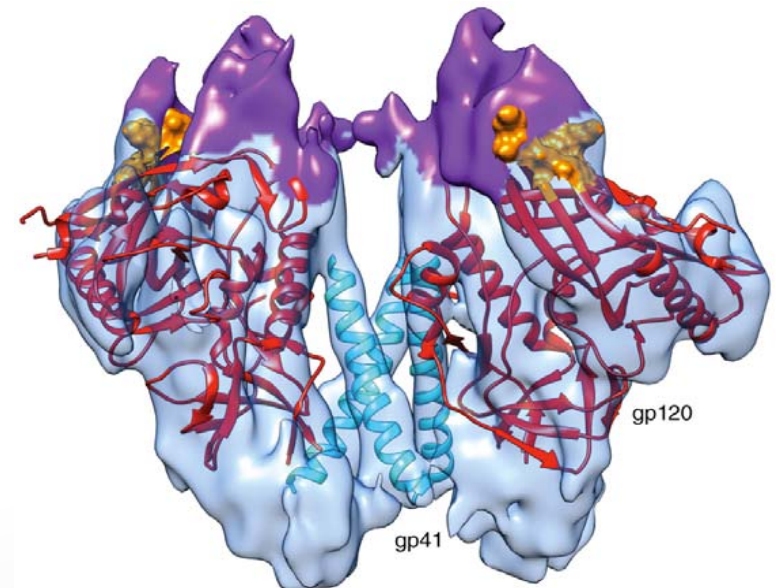


Why hasn't a vaccine been developed yet?

- Because of the nature of these glycoproteins, it is very hard to establish a vaccine that will cause the production of broadly neutralizing antibodies (bnAb)
- Both gp120 and gp41 are highly variable
- Glycosylation on gp120 protects conserved epitopes on both gp120 and gp41
- The heavy glycosylation also forms a cage-like structure around the gp41 trimer, causing the gp41 subunit to be very difficult to access.
- This protection restricts antibody access to the gp41 region, which provides a difficulty when producing a suitable vaccine.



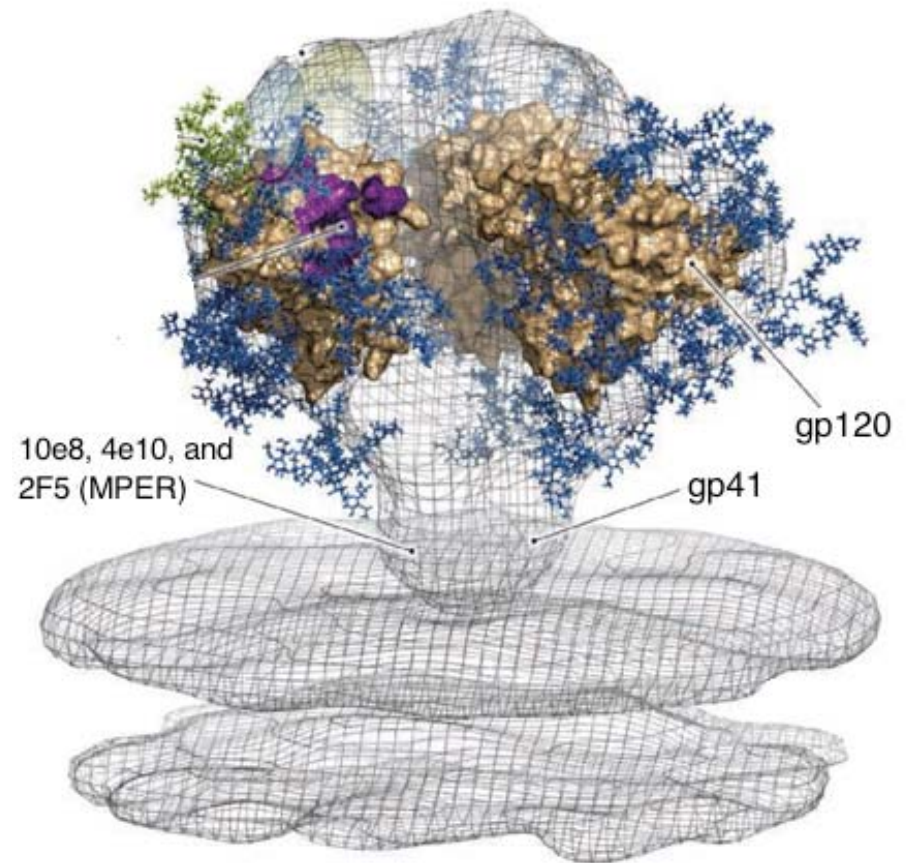
<http://home.ccr.cancer.gov/inthejournals/subramaniam.asp>



<http://www.nature.com/nsmb/journal/v20/n12/full/nsmb.2711.htm>

Some strains can be neutralized!

- Even though it is difficult, several antibodies have been isolated from patients that can neutralize a broad range of HIV to a varying degree of success.
- These antibodies include 2F5, 4e10, and 10e8, which all target the MPER in gp41.
- The most easily neutralized strains are tier 1 viruses. These viruses have typically been passaged in laboratories for easy use.
- Tier 2 and tier 3 viruses are harder to neutralize, but are the type of viruses that are found in most patients.



https://www.aidsreagent.org/program_info.cfm

So why can some strains be neutralized?

- Our lab focuses mainly on gp41 for several reasons:
 - gp41 is more highly conserved and the known bnAbs are concentrated in a very small region (MPER).
 - This small region allows for a minimization of the vaccine candidate size, which can also limit the amount of non-neutralizing antibodies produced.
- We would like to understand why tier 2 viruses are more difficult to neutralize with gp41-targeting bnAbs than tier 1 viruses.
- The information that will be gathered from this study has the potential to shed light on the biological reasons that tier 2 viruses are more resistant than tier 1 viruses.
- If we are able to understand how these differences prevent neutralizing antibodies from binding, we may be able to produce better immunogens able to guide the immune system to overcome these resistance-inducing mutations
- For this study, we used MN (tier 1, sensitive) and 6535 (tier 2, resistant) as representative viral strains

Original HIV-1 Envelope sequences

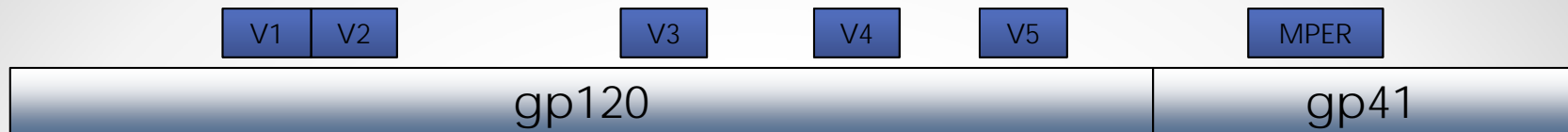
MN (tier 1A)= Sensitive to neutralization



6535 (tier 2)= resistant to neutralization



- Previous work identified a region of the gp41 envelope sequence that determined whether the strain was sensitive or resistant to neutralization
- This was accomplished by producing several chimera viruses using laboratory techniques such as digestion and ligation.
- Digestion was done using appropriate restriction sites to digest the original sequences into the desired fragments.
- Once the specific fragments were produced, ligation was used to create the following chimera viruses, which were used to narrow down the region in which the change(s) affecting neutralization resistance is located.



6535mMN:

MfeI



- 6535 at the gp120 region, breaks at the MfeI restriction site. The chimera is then MN at the gp41 region.
- We originally hypothesized that this chimera would be resistant, since the gp120 region should block access to the gp41 region of interest.

6535hMN:

HindIII



- 6535 at the gp120 region and the N terminus of the gp41 region, which includes the fusion peptide, N-heptad repeat and part of the C-heptad, of gp41.
- This breaks at the HindIII restriction site with the rest of the gp41 region represented by the MN strain.

6535aMN:

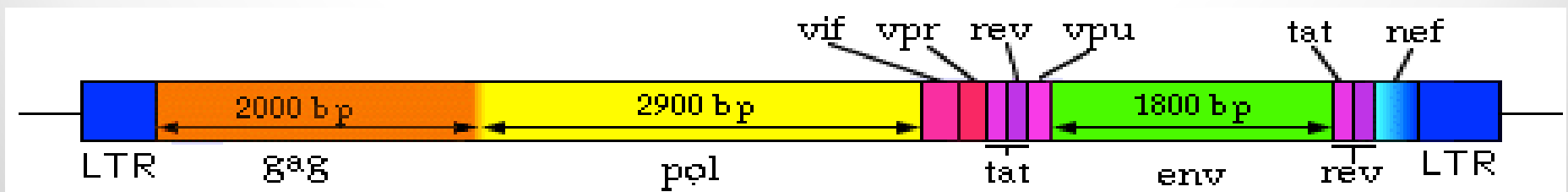
AvrII



- 6535 at the gp120 region and a small portion of the N terminus of gp41, which breaks at the AvrII restriction site. The remaining gp41 region is represented by the MN strain.
- It is important to note that in all three chimeras, the epitope that the brNabs bind to is always derived from the sensitive MN strain.

Pseudoviruses

- We do not work with infectious HIV-1 viral particles in the lab
- HIV-1 pseudoviruses infect a cell exactly like how HIV would normally infect, but when it comes to spreading and infecting more cells, it cannot.
- Pseudovirus genomes consist of two plasmids:
 - One plasmid contains everything except the envelope gene
 - The second plasmid contains only the envelope gene, but without the packaging signal.
- Without the packaging signal, the envelope gene is not packaged into the virion, so the virus cannot form an envelope and spread to other cells.
- Pseudoviruses produced for each mutant HIV-1 envelope genome, are used in neutralization assays, which measure the percent of neutralization different antibodies (2F5, 4e10, and 10e8) are able to produce.

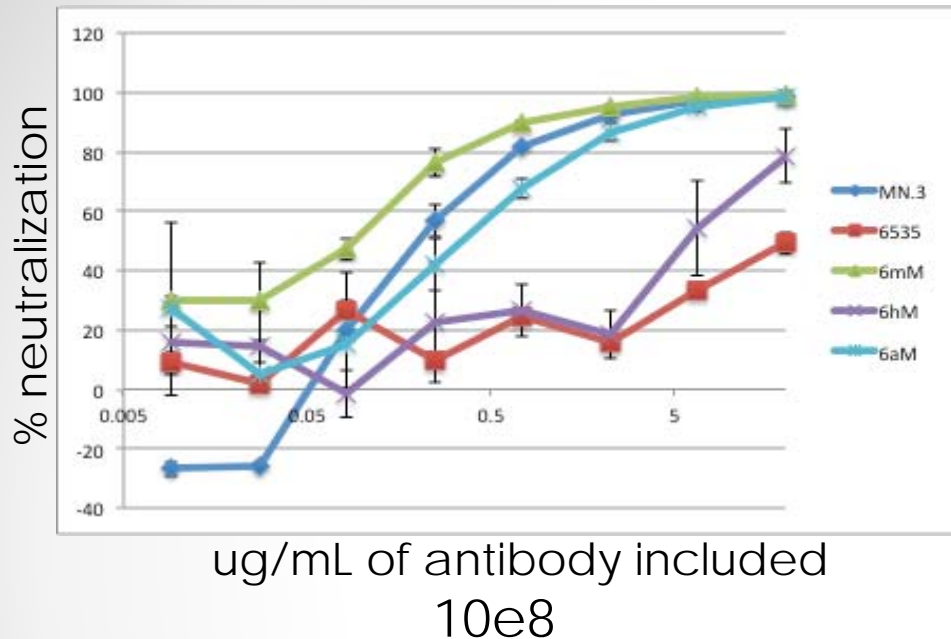


Neutralization Assays

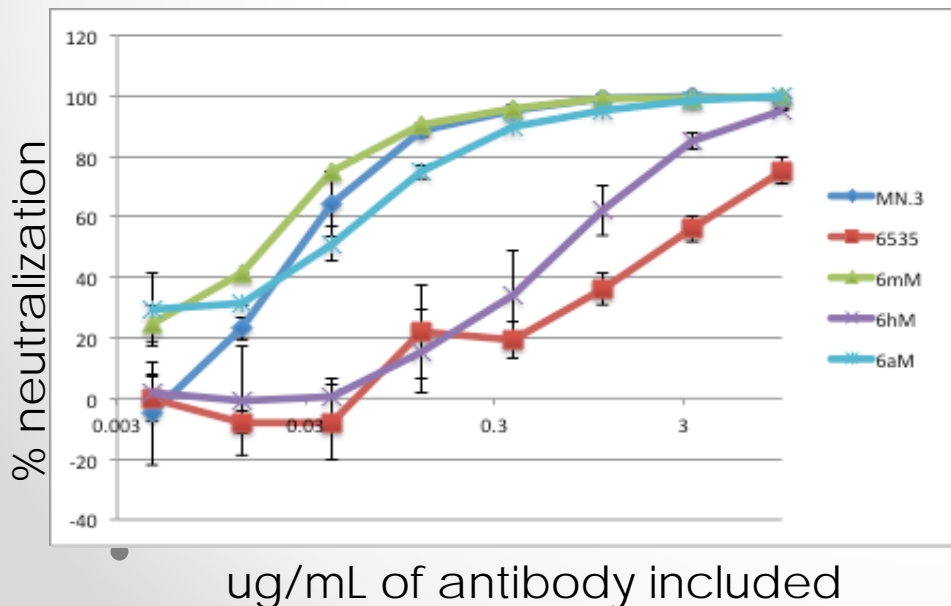
- Tzm-bl cells are used in these assays.
 - Tzm-bl cells are HeLa derived cells that express the CD4 receptor and the two coreceptors CCR5 and CXCR4, which allow the HIV pseudovirus to enter and infect the cells.
 - The cells also have a genetic insert that expresses B-galactosidase and luciferase, which are under the control of an HIV induced promoter.
 - Only when HIV is present, does the promoter turn on and drive B-galactosidase and luciferase expression.
- To perform this assay, a known amount of pseudovirus and several serial dilutions of monoclonal antibodies (mAb) are co-incubated without cells for one hour, giving the antibodies a chance to bind and neutralize the pseudovirus.
- Tzm-bl cells are then added to the pseudovirus and mAbs, and incubated for two days.
- To measure the amount of pseudovirus neutralized by the mAbs, luciferin is added, which will be cleaved by the luciferase enzyme and create a visible light signal.
 - This is measured in reflective light units (RLU).

Neutralization assays

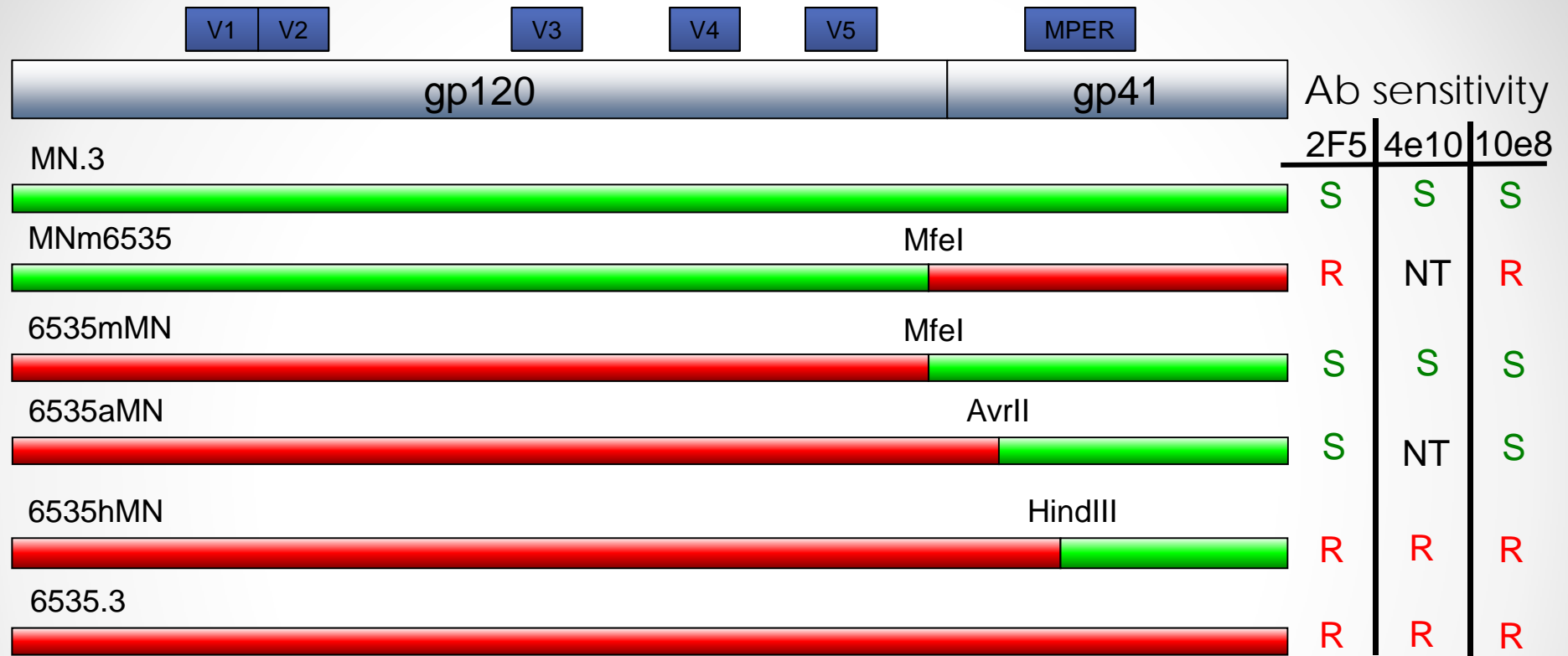
2F5



- These two graphs show the neutralization curve using the antibodies 2F5 and 10e8.
- The viral strain MN.3 (dark blue), and mutants 6535mMN (green) and 6535aMN (light blue) show neutralization sensitivity to both 2F5 and 10e8 antibodies
- The viral strains 6535 (red) and the mutant 6535hMN (purple) both show neutralization resistance to both 2F5 and 10e8 antibodies.
- These results would be reported as the point where the neutralization reaches 50%.
- These neutralization sensitive viral strains require a much lower amount of antibody for neutralization compared to the neutralization resistant strains.



Neutralization assay results



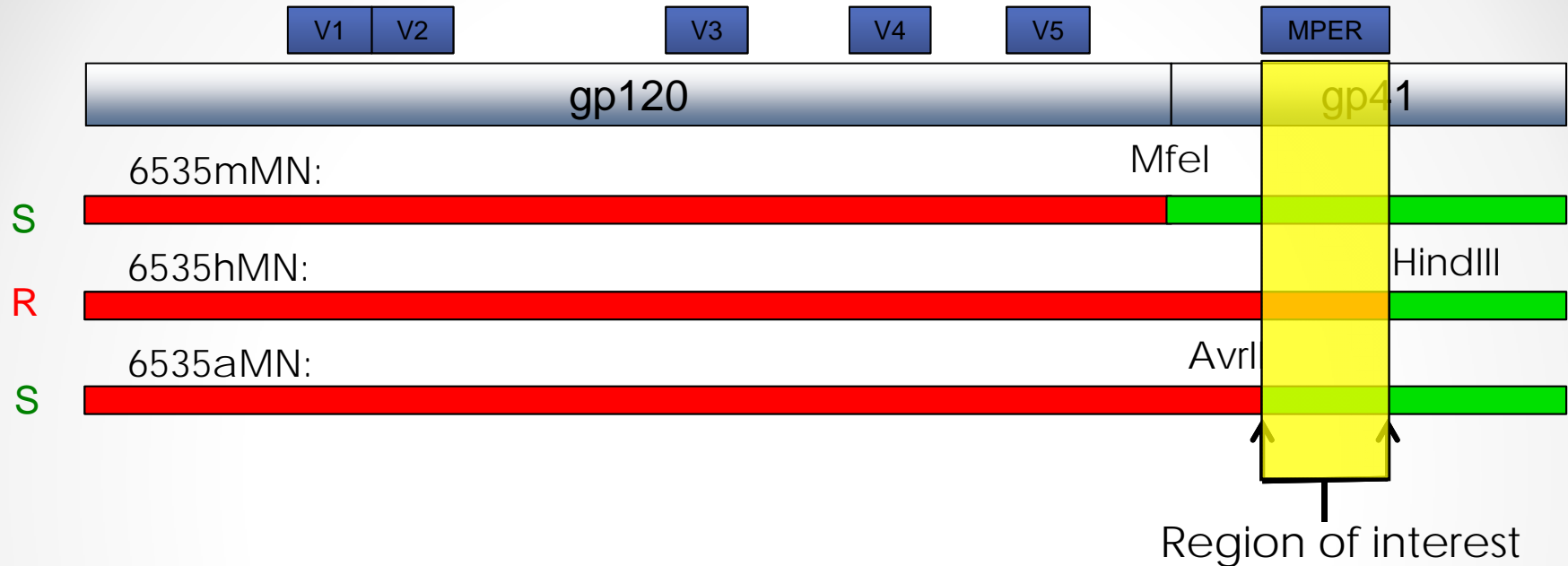
- Neutralization did occur in 6535mMN even though the gp120 was from the resistant strain, so gp120 had no effect on the neutralization
- MNm6535 remains resistant to neutralization confirms that the determining factor of sensitivity vs. resistance between these two strains to neutralization is located in the gp41 subunit
- 6535aMN and 6535hMN further narrow the region resulting the in difference between neutralization sensitivity

NT = Not tested

R = Resistant

S = Sensitive

Possible mutations affecting the neutralization resistance of HIV-1 6535 and MN strains



There are seven possible mutation within this region:

MN: AVERYLKDQQLLG**I**WGCSGKL
6535: AVERYLKDQQL**L**GFWGCSGKL

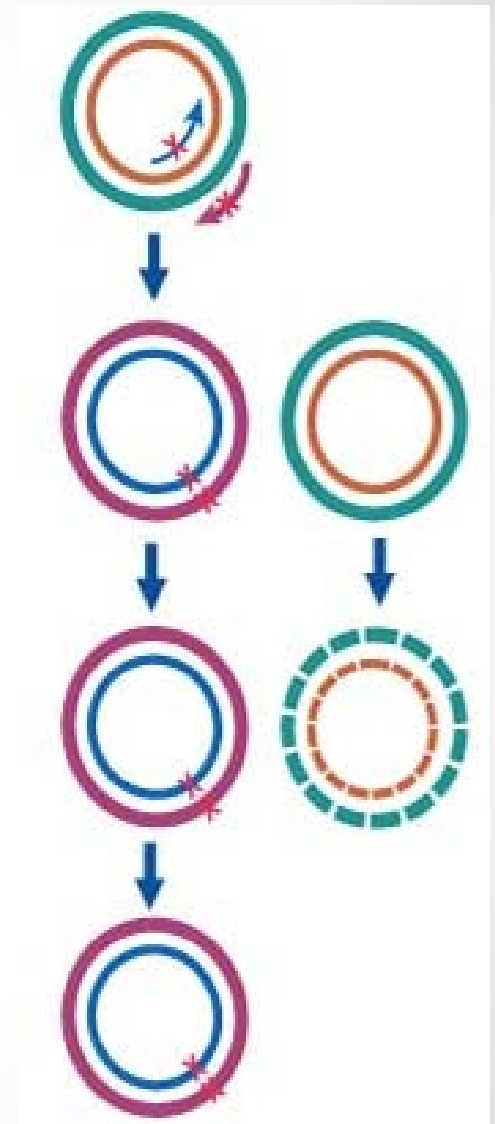
MN: ICTT**A**VPWNT**T**SWSNKSL**N****Y**IW
6535: ICTT**T**VPWNA**S**SWSNKSL**D****D**IW

MN: **D**NMTWME**W**EREIDNYTSL**I**Y
6535: **N**NMTWM**Q**WEREIDNYTSL**I**Y

HindIII

Site directed mutagenesis

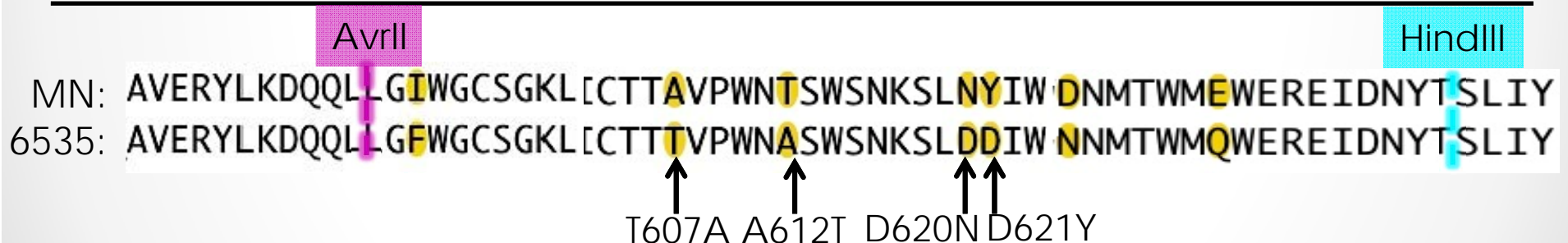
- Site directed mutagenesis allowed us to mutate a single amino acid to test whether or not each mutation affects the neutralization resistance of the virus.
- The first step is a simple PCR reaction with minor alteration and primers with a mismatched base pair to the original template DNA at the site of the desired mutation.
- This PCR reaction produces the desired mutant strand.
- The remaining parent strand is removed by digestion with Dpn1, which only targets methylated DNA.
- The final product is just the mutant strand, which is then transformed into competent cells that are able to repair the nick remaining from the linear PCR product.
- Extraction of the mutated genome is done, and a neutralization assay can then be performed using pseudoviruses expressing the mutated genome.



Production of Mutant Pseudovirus

- Before performing a neutralization assay, mutant pseudoviruses must be produced for each site directed mutant.
- However, when measuring the titers of the produced pseudoviruses we discovered that we did not have high enough yields to perform a neutralization assay.

Lanes	1	2	3	4	5	11
Mutant	T607A	A612T	D620N	D621Y	6535 wild type	Negative control
RLU per 25uL	2184	1309	446	1125	708	397



- The minimum reflective light units (RLU) needed to perform a neutralization assay is a 10 fold over the background.
- The background is 397 RLU per 25uL, so the minimum titer needed is about 4,000 RLU per 25uL
- These low numbers may be the result of poor cell quality during and after transfection.

Future Directions and Applications

- Currently we have confirmed the sequences of 5 out of the 7 possible mutants. These other two mutants will be confirmed as well
- We must still complete the pseudovirus production of all mutants, which will then be tested and assayed.
- The information that will be gathered from this study has the potential to shed light on why tier 2 viruses are more difficult to neutralize than tier 1 viruses.
- The differences responsible for the difference in neutralization resistance between viral strains MN and 6535 may be one of many mutations affecting resistance to gp41 targeting antibodies.
 - Multiple differences may act together to induce resistance.
- If we are able to understand how these differences prevent neutralizing antibodies from binding, we may be able to produce better immunogens able to guide the immune system to overcome these resistance-inducing mutations

Thank you!